(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 January 2001 (25.01.2001)

PCT

(10) International Publication Number
WO 01/05819 A1

(51) International Patent Classification[7]: C07K 7/64, A61K 38/08, C08G 65/333

(21) International Application Number: PCT/KR99/00379

(22) International Filing Date: 15 July 1999 (15.07.1999)

(25) Filing Language: English

(26) Publication Language: English

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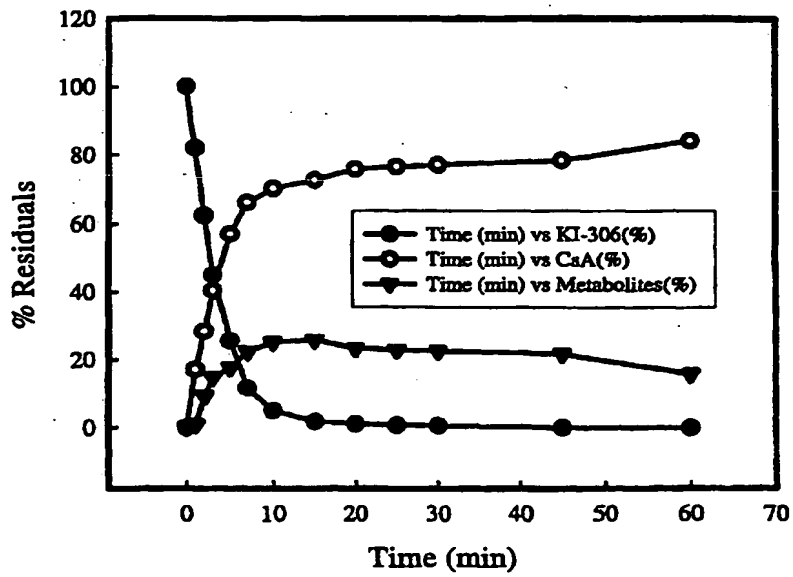
(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

[Continued on next page]

(54) Title: NOVEL WATER SOLUBLE-CYCLOSPORINE CONJUGATED COMPOUNDS



(57) Abstract: The present invention relates to a water soluble polymer-cyclosporine conjugated compound, more specifically, to a drug-delivery to cyclosporine wherein the drug is chemically bound to a water-soluble polymeric or macromolecular carrier that renders the drug water-soluble and more bioavailable.

WO 01/05819 A1

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NOVEL WATER SOLUBLE-CYCLOSPORINE CONJUGATED COMPOUNDS

TECHNICAL FIELD

The present invention relates to a water soluble polymer-cyclosporine conjugated compound. More specifically, the present invention relates to a drug-delivery to cyclosporine wherein the drug is chemically bound to a water-soluble polymeric or macromolecular carrier that renders the drug water-soluble and more bioavailable. In another aspect, the present invention relates to water-soluble cyclosporine prodrugs that recover their biological activity when it is hydrolyzed from the carrier molecules.

BACKGROUND ART

Cyclosporine is a peptide compound having a unique structure consisting of 11 poly-N-methylated amino acids and has been known to have useful pharmacological activities. Especially, immunosuppressive properties of systematically administered cyclosporine are used in therapy or during organ transplants or bone marrow transplants. It is also applicable to the treatment of broad range of autoimmune diseases of inflammatory etiology and also to the antiparasitic treatment. Cyclosporine is used, for example, for the treatment of rheumatic diseases (rheumatoid polyarthritis), hematological disease (aplastic anemia, idiopathic thrombocytopenia), gastric disorders (ulcerating colitis, crons disease), dermatic disease (psoriasis, sclerodermia) and eye diseases (uveitis). Also topical applications have been tested, for example,

in treatment of psoriasis, uveitis and alopecia.

Cyclosporine is highly lipophilic, poorly water-soluble and, therefore, typically supplied as an olive oil or peanut oil solution for clinical use. However, the bioavailability of cyclosporine from such oily solution is very low and gives rise to great intersubject variation with systemic availability ranging from 4 to 25% (see Takada, K. et al, *J. Pharmacobio-Dyn.*, **11**: 80-7, 1988). The bioavailability of cyclosporine has been reported to depend on food, bile and other interacting factors (see Fahr, A., *Pharmacokinetics*, **24**: 472-95, 1993). In a recent study in which a microemulsion preparation of cyclosporine was administered locally to different parts of the small and large intestine (duodenum, jejunum, ileum and colon descendents), cyclosporine was found to be absorbed predominantly in the small intestine (see Drewe, J., et al, *J. Clin. Pharmac.*, **33**: 39-43, 1992).

Cyclosporine has a low bioavailability and tissue-availability and thus, should be administered in an excessive amount. Therefore, the administeration of excess cyclosporine may frequently has undesirable side effects such as nephrotoxicity, hypertension, hyperkalemia, hyperurikemia, hepatotoxicity, anemia, gastrointestinal intolerance, tremor and parestesia. The most frequent side effect is usually renal dysfunction. Acute cyclosporine nephrotoxicity is dose-dependent. There is a correlation with the blood level and a decrease in the dose or discontinuation of cyclosporine therapy leads to an improvement. However, progressive and irreversible damage of kidneys was reported in patients with transplants.

Cyclosporine is neutral, insoluble in water and n-hexane, but very soluble in all organic solvents. Due to the poor solubility of aqueous cyclosporine solution, the formulations require a surfactant to solubilize the drug. The pharmaceutical preparation of cyclosporine (Sandimmune®) which is used clinically is prepared in the form of solution, used for injection or oral administration, or a soft capsule filled with the solution. Formulations for oral and intravenous administrations of cyclosporine are prepared in the form of microemulsion.

A liquid microemulsion formulation is prepared by combining cyclosporine with a surfactant, an oil and a cosurfactant (see U.S. Patent No. 4,388,307). The microemulsion of cyclosporine is consisted with ethanol as a cosurfactant, a vegetable oil and a transesterified product of a natural vegetable oil triglyceride and a polyalkylene glycol as a surfactant to form the liquid formulation. Injection preparation containing a nonionic surfactant such as a Cremophor EL can develop the analphylaxis reaction to a few cases (see Lorence, W., et el, *Agents and actions*, **12**: 64-80, 1982). Also, the addition of nonaqueous solvents such as ethanol, propylene glycol or polyethylene glycol 400 needs to be considered for parenteral administration. Using such organic solvent has a problem such as hemolysis and local irritation at injection site.

In preparing oral formulation, a soft capsule filled with the microemulsion solution as a main component has a few drawbacks during the absorption process. When the oily components are contacted with an aqueous solution in mouth or intestine, the drug component may be often separated as

a solid, thereby reducing its bioavailability to a level of below 30%. Moreover, in case of a long period storage, cyclosporine tends to be crystallized as the ethanol content decreases by evaporation of ethanol; and patients suffer from the unpleasant odor of the ethoxylated castor oil.

Even though the other processes may have achieved with some success in improving the stability of the formulation by minimizing the ethanol content therein, there still remain various deficiencies. For example, the use of surfactants having a complicated composition is not practically easy or suitable for the development of the formulation; in case of liposome (WO 90-00389) the whole process is complicated and the reproducibility of particle size or inclusion rate is hard to control; and the use of polymeric polysaccharide (German Patent No. 293,499) has disadvantage that the total volume of the formulation may be too bulky for administration. Further, the prior art methods still fail to produce cyclosporine containing composition which have a satisfactory dissolution rate in an aqueous solutiuon.

In order to avoid the problems such as the toxicity of surfactant and solvent in preparing the formulation, if such insoluble drugs is attached to water-soluble macromolecules to act as carriers, it will greatly reduce these problems and will be suitable for parenteral and oral administrations. The delivery of cyclosporine attached to a polymeric water-soluble carrier such as polyethylene glycol(PEG) has never been considered up to now. Polyethylene glycol is a linear or branched, neutral polymer having various molecular weight range and is soluble in water and methylene chloride. PEG having a molecular weight of less than 1000 are viscous and colorless liquid; PEG

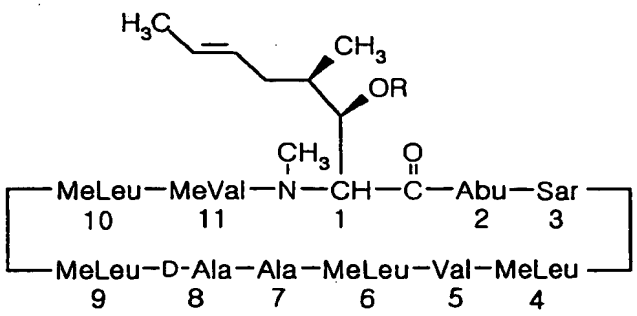
having higher molecular molecular weight is waxy and white solids. The melting point of the solid is proportional to the molecular weight, approaching a plateau at 67℃. The molecular weights from a few hundred to about 20,000 are commonly used in biological and biotechnolgical applications.

One of the interests in the biomedical areas is the fact that PEG is nontoxic and was approved by Food and Drug Administration(FDA) in the United States for internal consumption. PEG is widely used for the synthesis of drug and for a wide variety of cosmetic and personal care products. One of the most extensively studied drug-delivery technologies involves the covalent linkage of the polymer monomethoxypoly(ethylene glycol) (mPEG) to the surface of proteins (see Harris, M. J., Poly(ethylene Glycol) Chemistry, Biotechnical and Biomedical Applications).

Therefore, the present inventors have developed a novel water-soluble polymer-cyclosporine conjugated compound which is formed by chemically combining cyclosporine to a water-soluble carrier. The compounds of this invention are water soluble prodrugs of cyclosporine which are useful as immunosuppressant, antiinflammatory, antifungal and antiproliferative agents.

DISCLOSURE OF INVENTION

Thus, the present invention relates to a water-soluble polymer-cyclosporine conjugated compound represented by the following formula (I):



(I)

in which

R represents a group of formula (a), (b) or (c):

(a) $-C(=O)-OCH_2XC(=O)-(CH_2)_mC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$,

(b) $-C(=O)-OCH_2XC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$, or

(c) $-C(=O)-OCH_2X-CH_2CH_2-(OCH_2CH_2)_n-OCH_3$,

X represents O, S, or NH,

m is an integer of from 1 to 6, preferably 1 to 3, and

n is an integer of 10 to 460, preferably 10 to 22, most preferably 90 to 120.

The present invention also relates to a process for preparing a water-soluble polymer-cyclosporine conjugated compound of the above formula (I).

The present invention further relates to a pharmaceutical composition containing as an active ingredient a conjugated compound of the above formula (I) together with a pharmacetically acceptable carrier.

The present invention further relates to a method of treating transplantation rejection or graft vs. host disease in a mammal in need thereof, which comprise administering an effective amount of a conjugated

compound of the above formula (I) to said mammal.

The present invention further relates to a method of treating a fungal infection in a mammal in need thereof, which comprises administering an effective amount of a conjugated compound of the above formula (I) to said mammal.

The present invention further relates to a method of treating rheumatoid arthritis in a mammal in need thereof, which comprises administering an effective amount of a conjugated compound of the above formula (I) to said mammal.

The present invention further relates to a method of treating a retenosis in a mammal in need thereof, which comprises administering an effective amount of a conjugated compound of the above formula (I) to said mammal.

The present invention further relates to a method of treating a pulmonary inflammation in a mammal in need thereof, which comprises administering an effective amount of a conjugated compound of the above formula (I) to said mammal.

BRIEF DESCRIPTION OF DRAWINGS

For a through understanding of the nature and objects of the invention, reference should be made to the following detailed description

taken in connection with the accompanying drawings in which:

Figure 1 shows a concentration-time profile of the hydrolysis of the conjugated compound of the present invention (KI-306, Cyclosporine 3'-methoxypropyleneglycole 5000-succinyloxymethyloxy carbonate ester) to cyclosporine, as the parent drug, in human liver at 37℃.

Figure 2 is a linear graph showing the first-order plot of the hydrolysis of the conjugated compound (KI-306) of the present invention to cyclosporine in human liver homogenate at 37℃.

Figure 3 is a linear graph showing the first-order plot of KI-306 disappearance in rat blood as a function of time after i.v. injection (7 mg/kg) into rats.

Figure 4 is a graph showing the concentration-time profile in rat whole blood concentration over time after cyclosporine prodrug (KI-306) in saline solution and Sandimmune Neoral Oral Solution by oral administration.

BEST MODE FOR CARRYING OUT THE INVENTION

The general chemical structure of cyclosporine is a conjugated compound of formula (I) in which R represents hydrogen(H). Of the compounds of this invention, those which m is 1 to 3 and n is 10-140 are preferred. Those which n is 10-120 is most preferred.

Among the conjugated compounds of the formula(I), those wherein R represents one group selected from the following formulas are preferred:

- $-C(=O)-OCH_2OC(=O)-(CH_2)_mC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$;
- $-C(=O)-OCH_2SC(=O)-(CH_2)_mC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$;
- $-C(=O)-OCH_2NHC(=O)-(CH_2)_mC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$;
- $-C(=O)-OCH_2OC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$;
- $-C(=O)-OCH_2SC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$;
- $-C(=O)-OCH_2NHC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$;
- $-C(=O)-OCH_2OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$;
- $-C(=O)-OCH_2SCH_2CH_2-(OCH_2CH_2)_n-OCH_3$; or
- $-C(=O)-OCH_2NHCH_2CH_2-(OCH_2CH_2)_n-OCH_3$.

Water-soluble polymers such as polyethylene glycol(PEG), and monomethoxy polyethylene glycol(mPEG) are utilized to bind to poorly aqueous-soluble drugs and increase a water solubility of the drugs. Therefore, cyclosporine, sparingly soluble drug, is utilized for covalent linking with the carrier polymers to be dissolved in water.

The water soluble polymer-cyclosporine conjugated compounds according to the present invention, i.e., compounds of formula (I) that are esterified at the 3'-OH of cyclosporine, can be prepared by esterifying a compound of the following formula (II) with a base such as pyridine to give a compound of the following formula (III) and then reacting the resulting compound (III) with polyethylene glycol derivatives, compounds of the following formulas (IV), (V) or (VI), in the presence of sodium iodide, potassium carbonate or crown ether, respectively:

$Y\text{-}CH_2O\text{-}C(=O)\text{-}O\text{-}C(=O)\text{-}OCH_2\text{-}Y$ (II)

$CsA\text{-}O\text{-}C(=O)\text{-}OCH_2\text{-}Y$ (III)

$OCH_3\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2O\text{-}C(=O)\text{-}(CH_2)_m\text{-}C(=O)\text{-}XH$ (IV)

$OCH_3\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2O\text{-}C(=O)\text{-}XH$ (V)

$OCH_3\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2O\text{-}XH$ (VI)

in which

Y represents a leaving group such as halogen,

X represents O, S or NH,

m is an integer from 1 to 6, and

n is an integer of 10 to 460, preferably 10 to 220, most preferably 90 to 120.

In view of the results of enzyme kinetic study, cyclosporine conjugate of the present invention has the same pharmacological usage as cyclosporine itself. That is, the conjugated compound of the present invention can be used for the treatment of transplantation rejection such as kidney, heart, liver, lung, bonemarrow, pancreas, cornea, small bowel, and skin allografts, and heart valve xenografts, for the treatment or inhibition of graft vs. host diseases, for the treatment or inhibition of autoimmune disease such as lupus, rheumatoid arthritis, diabetes mellitus, myasthenia, dermatitis, eczema, seborrhea, inflammatory bowl disease, pulmonary inflammation (including asthma, chronic obstructive pulmonary disease, emphysema, acute respiratory disease syndrome, bronchitis, and the like) and eye uveitis. In view of the pharmacological activities of cyclosporine, the compound of present invention are also considered to have antifungal and antiproliferative activities, and therefore, also useful in the treatment in fungal infection and

hyperproliferative vascular disease such as restenosis and atherosclosis. When used for this purpose, the compound of the present invention can be administered prior to the procedure, during the procedure, subsequent to the procedure, or any combination of the above.

When administered for the treatment or inhibition of the above disease state, the conjugated compounds of the present invention can be administered orally, parenterally, intranasally, transdermally, topically, intravaginally or rectally. The conjugated compounds of the present invention are particularly advantageous as immunosuppressive, antiinflammatory, antifungal, and antiproliferative agents because of their water solubility.

It is contemplated that when the conjugated compounds of the present invention are used as an immunosuppressive or antiinflammatory agent, they can be administered in conjunction with one or more other immunoregulatory agents. Such other immunoregulatory agents include azathioprine, corticosteroids, such as prednisolone and methylprednisolone, cyclophosphamide, rapamycin, tacrolimus, OKT-3, ATG, etc., but they are not limited to these. By combining the conjugated compounds of the present invention with such other drugs or agents for treating immunosuppression or inflammatory conditions, the desired effect may be achieved by the lesser dosage of each of the agents.

The conjugated compound of the present invention can be formulated alone or, if necessary, together with a pharmaceutically acceptable carrier. The pharmaceutical carrier may be solid or liquid. A solid carrier may include

one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or tablet-disintegrating agents, and it can also be an encapsulating material. In powders, the carrier is a finely divided solid that can be mixed with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size as desired. Suitable solid carrier include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, methyl cellulose, sodium casrboxymethyl cellulose (sodium CMC), polyvinylpyrrorolidone (povidone), low melting waxes and ion-exchange resins.

Liquid carrier are used in preparing solutions, suspensions, emulsions, syrups, elixers and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as buffers, preservatives, sweeteners, flavoring agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additive as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, lecithins, and oils (e.g. coconut oil and arachis oil).

For parenteral administration, the carriers are used in sterile liquid

form compositions. The liquid carrier for pressurized composition (in the form of an aerosol) can be halogenated hydrocarbon or other pharmaceutically acceptable propellant. Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by, for example, intramuscular, intraperitoneal or subcutaneous injection. Sterile solutions can also be administered intravenously. The conjugated compound of the present invention can also be administered orally either in liquid or solid composition form.

The conjugated compound of the present invention may be administered rectally in the form of a conventional suppository. For administration by intranasal or intrabronchial inhalation or insufflation, the conjugated compounds of the present invention may be formulated into an aqueous or partially aqueous solution, which can be utilized in the form of aerosol. The conjugated compound of the present invention may also be administered transdermally through the use of a transdermal patch containing the active compound and a carrier that is inert to the active compound of the present invention, is non toxic to the skin and allows delivery of the agent for systemic absorption into the blood stream via the skin. The carrier may take a number of forms such as creams and ointments, pasts, gels, and occulsive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes are comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may be possible. A variety of occlusive devices may be used to release the active ingredient into the blood stream such as a semipermeable membrane covering a reservoir containing the

active ingredient with or without a carrier, or a matrix containing the active ingredient.

In addition, the conjugated compounds of the present invention may be employed as a solution, cream or lotion by formulation with pharmaceutically acceptable vehicles containing 0.1-5%, preferably 2%, of the active compound which may be applied to a fungally affected area.

The dosage requirements may vary with the particular compositions employed, the route of administration, the severity of the symptoms presented and the particular subject being treated. Preferably, the pharmaceutical composition is in unit dosage form, e.g. as tables or capsules. In such form, the composition is sub-divided in unit dose containing appropriate quantities of the active ingredient. The unit dosage forms can be packaged compositions, for example, packed powders, vials, amples, prefilled syringes or sachets containing liquids. The unit dosage form can be, for example, packaged powders, vials, ampules, prefilled syringes or sachets or tablet itself, or it can be the appropriate number of any such composition in package form.

Hereinafter, the present invention will be more specifically explained on the basis of the following examples. However, it should be understood that the technical scope of the invention is not limited by these examples in any manner.

Example 1

Synthesis of CsA-Chloromethyl Carbonate

After chloroformic acid (400 $\mu\ell$, 4.2 mmol) was added dropwise to a stirred mixture of anhydrous cesium carbonate (685 mg, 2.10 mmol) in dichloromethane or tetrahydrofuran (5 mL) and the reaction mixture was stirred for 12 hours at room temperature. Cyclosporine A (CsA 100 mg, 0.083 mmol) and pyridine (1 mL) were added to the reaction mixture and the mixture was stirred for 12 hours at room temperature. After stirring for 12 hours, ether (50 mL) was added to the mixture. The resulting precipitate was filtered off and the filtrate was evaporated *in vacuo*. The crude product was triturated from ether/petroleum ether(2:1) to yield (97 mg, Yield: 90%) of CsA-chloromethyl carbonate.

IR(KBr, cm^{-1}) 1760(ester); ^1H NMR(500 MHz, $CDCl_3$) δ 5.70(2H, dd, J = 58.98 and 6.33 Hz, $ClCH_2OCOO$); ^{13}C NMR(125 MHz, $CDCl_3$) δ 173.7, 173.4, 173.1, 172.8, 171.6, 171.2, 170.9, 170.8, 170.0, 169.9, 167.6, 153.5(C=O, 12 units); FAB-MS(m/z) 1294(M^+).

Example 2

Synthesis of Cyclosporine-mPEG Conjugate, KI-306

Method 1: A mixture of CsA-chloromethyl carbonate (100 mg, 0.077 mmol), mPEG-succinate 5000 (385 mg, 0.077 mmol), and Cs_2CO_3 (50 mg, 0.154 mmol) in anhydrous acetonitrile (10 mL) was stirred at 85℃ for 24 hours. After diluting with acetonitrile (5 mL), the insoluble material was filtered off. The filtrate was evaporated *in vacuo*. The crude product was

triturated from CH_2Cl_2/ether(1 : 5) to yield 350 mg of CsA-mPEG conjugate (purity = 30%, Prep. HPLC).

The unreacted mPEG was removed by a preparative HPLC system. MS(MALDI/TOF) shows a mean molecular weight of 6377 for the product and 5117 for the starting mPEG-succinate 5000. The difference in mass(1260) matched the cyclosporine chloromethyl carbonate.

^{1}H NMR(300 MHz, $CDCl_3$) δ 5.77(2H, q, J = 5.67 Hz, $OCOCH_2OCO$); ^{13}C NMR(75 MHz, $CDCl_3$) δ 173.59, 173.27, 172.91, 172.71, 171.87, 171.46, 171.17, 170.87, 170.76, 170.57, 169.98, 169.81, 167.58, 154.07(C=O, 14 units); MS(MALDI/TOF) m/z 6377 (mean MW)

HPLC Method:

1. Column: Waters C8, 3μ, 4.6 x 150 mm
2. Mobil Phase: A= 40% water
   B= 60% ACN
3. Flow Rate: 1.0 mL/min
4. Column Temp.: 65℃
5. Detection: UV at 215 nm
6. Retention Time: 17.85 min

Method 2: A mixture of CsA-chloromethyl carbonate (142.5 mg, 0.11 mmol), mPEG-succinate 5000 (500 mg, 0.1 mmol), NaI (33 mg, 0.22 mmol), Cs_2CO_3 (50 mg, 0.15 mmol), and crown ether (26.4 mg, 0.1 mmol) in benzene 50 mL(or THF, toluene) was stirred at 80℃ for 24 hours. After

diluting with ethylacetate (20 mL), the insoluble material was filtered off. The filtrate was evaporated *in vacuo*. The crude product was crystallized from CH_2Cl_2/ether(1:5) to yield 480 mg of CsA-mPEG conjugate (purity = 75%, Prep. HPLC)

Method 3: A mixture of CsA-chloromethyl carbonate (142.5 mg, 0.11 mmol), mPEG-succinate 5000 (500 mg, 0.1 mmol), NaI (33 mg, 0.22 mmol), K_2CO_3 (20.7 mg, 0.15 mmol), and crown ether (26.4 mg, 0.1 mmol) in benzene 50 mL(or THF, toluene) was stirred at 80℃ for 24 hours. After dilution with ethylacetate (20 mL), the insoluble material was filtered off. The filtrate was evaporated *in vacuo*. The crude product was triturated from CH_2Cl_2/ether(1:5) to yield 465 mg of CsA-mPEG conjugate. (purity = 73%, Prep. HPLC)

Example 3

Synthesis of Cyclosporine-mPEG Conjugate, KI-312

A mixture of CsA-chloromethyl carbonate (142.5 mg, 0.11 mmol), mPEG-glutamate 5000 (500 mg, 0.1 mmol), NaI (33 mg, 0.22 mmol), Cs_2CO_3 (50 mg, 0.15 mmol), and crown ether (26.4 mg, 0.1 mmol) in benzene(50 mL) THF, or toluene was stirred at 80 ℃ for 24 hours. After dilution with ethylacetate (20 mL), the insoluble material was filtered off. The filtrate was evaporated *in vacuo*. The crude product was triturated from CH_2Cl_2/ether(1:5) to yield 473 mg of CsA-mPEG conjugate (purity = 70%, Prep. HPLC)

The unreacted mPEG was removed by a preparative HPLC system. MS(MALDI/TOF) shows a mean MW of 6377 for the product and 5117 for the starting mPEG-glutamate 5000. The difference in mass(1260) matched the cyclosporine chloromethyl carbonate.

^{1}H NMR(300 MHz, $CDCl_3$) δ 5.75(2H, q, J = 5.67 Hz, $OCOCH_2OCO$); ^{13}C NMR(75 MHz, $CDCl_3$) δ 173.59, 173.27, 172.91, 172.71, 172.62, 171.47, 171.18, 171.10, 170.87, 170.76, 169.98, 169.81, 167.58, 154.10(C=O, 14units); MS(MALDI/TOF) m/z 6377 (mean MW)

Example 4

Hydrolysis of the prodrug (KI-306) in human liver homogenate at 37℃

To prove that the conjugated compound as synthesized according to the above method is decomposed in the body to produce cyclosporine, the enzymatic hydrolysis test is conducted using human liver homogenate at 37℃. Specifically, 3.0 g of human liver is introduced into 3.0 mL of 0.1 M phosphate buffer (pH 7.4), homogenized on ice and then centrifuged for 10 minutes. The supernatant is transferred to another tube. The test solution is prepared by dissolving 51.6 mg (10 mg CsA equivalent/mL) of the conjugated compound (Example 2) in 1.0 ml of 0.1 M phosphate buffer (pH 7.4).

A 90 $\mu\ell$ of the supernatant is introduced into each Eppendorf tube and maintained at 37 ℃. Then, 10 $\mu\ell$ of the test solution which is previously warmed to 30℃ is added thereto. The reaction mixture in each

tube is stirred for 5 seconds and 300 $\mu\ell$ of acetonitrile is added at the given interval (0, 1, 3, 5, 7, 10, 15, 30, 45, 60, 90, 120 minutes) and then the mixture in the tube is stirred for one minute. The tube is centrifuged at 13,000 rpm for 10 minuted and then stored on ice. In the tube, the final theroetical concentration of the conjugate is 1.29 μg/mL (250 μg/mL CsA equivalent).

Each 10 $\mu\ell$ of the sample solution is analyzed by means of HPLC. For HPLC analysis, a reverse-phase column waters C8, 3 μ (4.6x150 mm), is used. The mixture of 40% water-60% acetonitril solution is used as the isocratic mobile phase. The flow rate is 1.0 mL/min and the effluent is monitored at 215 nm and at 65 ℃.

As shown in Figure 1, the conjugated compound of the present invention is decomposed in human liver homogenate to produce the active material, cyclosporine A. The conjugate is linearly decomposed with a first-order kinetics as shown in Figure 2. Thus, it can be noted that the conjugate of the present invention is converted again into cyclosporine by the action of enzymes in human liver homogenate, and the hydrolysis half-life of the conjugate is 2.2 minutes at 37 ℃. This is ideal for the prodrug of cyclosporine. However, a demonstration of non-enzymatic conversion in pH 7.4 phosphate buffer is provided by the fact that the half-life of Example 2 is 21 hours at 37℃.

Example 5

Pharmacokinetic study

The pharmacokinetic study of the present invention (Example 2) in comparison to the commercial product (Sandimmune Neoral Solution) was carried out after single oral dose. Sprague-Dawley rats weighing 220 ± 30 g were used in this study. The rats were fasted overnight but were allowed free access to water.

Each rat received 7 mg/kg of CsA equivalent dose in one of the following dosage forms:

(1) CsA commercial (Sandimmune Neoral, Novatis Pharm. Ag, Basel, Switzerland),

(2) The prodrug of Example 2 (KI-306) dissolved in saline solution immediately prior to dosing.

The oral solutions were administered using oral sonde while the marginal tail vein was used for the i.v. dosing with the aid of implanted cannula for collecting blood samples. The Blood sample (200~250 $\mu\ell$) were collected in Eppendrof tube treated with heparine and taken at designed time intervals. The blood sample was pretreated with acetonitril and the supernatant organic layer was subjected to HPLC analysis. It was noted in case of orally administered Example 3 (KI-306) that only cyclosporine was detected, not for Example 2 (KI-306). As shown in Figure 3, the disappearance of KI-306 in rat blood by i.v. injection was found to be a half-life of 2.5 minutes. This data is good agreement with that in human liver homogenate with 2.2 minutes.

Descriptive pharmacokinetic parameters of two compartment models with lag time were obtained by using WinNonlin Program. The results as shown in Table I and Figure 4 demonstrated that greater bioavailability of the present invention (Example 2) is achieved as compared with Sandimmune Neoral Solution, as indicated by the 65% higher AUC values of Example 2.

Table I.

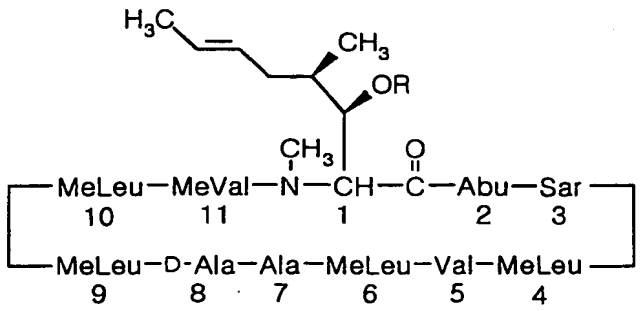
PK Parameter	KI-306		Neoral	
	Mean ($\mu g \cdot h/mL$)	CV (%)	Mean ($\mu g \cdot h/mL$)	CV (%)
AUC	32.79	19.85	21.40	10.03
C_{max}	1.77	4.40	1.08	3.26
T_{max}	1.43	11.35	2.55	16.08

It is established from the above Table I that the water soluble polymer-cyclosporine conjugated compound according to the present invention exhibits 65% higher AUC value than the conventional Neoral.

As set forth above, the water soluble polymer-cyclosporine conjugated compound of the present invention has a higher bioavailability. Therefore, the compound of the present invention may, even if administered in the lesser amount, achieve the equivalent or superior to the conventional drugs and may greatly reduce side effects such as nephrotoxicity, hypertension, hyperkalemia and the like.

WHAT IS CLAIMED IS:

1. A water soluble polymer-cyclosporine conjugated compound represented by the following formula (I):



(I)

in which R represents a group of formula (a), (b) or (c):

(a) $-C(=O)-OCH_2XC(=O)-(CH_2)_mC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$

(b) $-C(=O)-OCH_2XC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$, or

(c) $-C(=O)-OCH_2X-CH_2CH_2-(OCH_2CH_2)_n-OCH_3$,

X represents O, S, or NH,

m is an integer of from 1 to 6, and

n is an integer of 10 to 460.

2. The conjugated compound of claim 1 wherein m is 1 to 3.

3. The conjugated compound of claim 1 wherein n is 10 to 220.

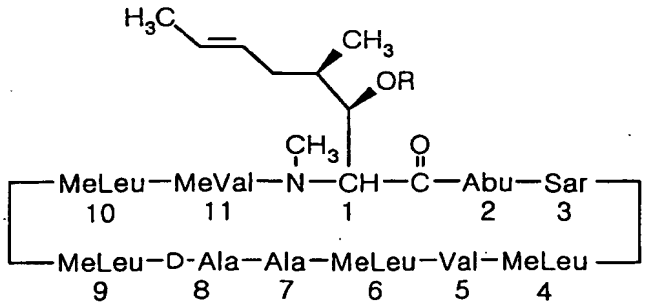
4. The conjugated compound of claim 1 wherein n is 90 to 120.

5. The conjugated compound of claim 1 wherein R represents one group

selected from the following formulas:

- -C(=O)-OCH_2OC(=O)-$(CH_2)_m$C(=O)-OCH_2CH_2-$(OCH_2CH_2)_n$-OCH_3;
- -C(=O)-OCH_2SC(=O)-$(CH_2)_m$C(=O)-OCH_2CH_2-$(OCH_2CH_2)_n$-OCH_3;
- -C(=O)-OCH_2NHC(=O)-$(CH_2)_m$C(=O)-OCH_2CH_2-$(OCH_2CH_2)_n$-OCH_3;
- -C(=O)-OCH_2OC(=O)-OCH_2CH_2-$(OCH_2CH_2)_n$-OCH_3;
- -C(=O)-OCH_2SC(=O)-OCH_2CH_2-$(OCH_2CH_2)_n$-OCH_3;
- -C(=O)-OCH_2NHC(=O)-OCH_2CH_2-$(OCH_2CH_2)_n$-OCH_3;
- -C(=O)-$OCH_2OCH_2CH_2$-$(OCH_2CH_2)_n$-OCH_3;
- -C(=O)-$OCH_2SCH_2CH_2$-$(OCH_2CH_2)_n$-OCH_3; or
- -C(=O)-$OCH_2NHCH_2CH_2$-$(OCH_2CH_2)_n$-OCH_3.

6. A process for preparing a water soluble polymer-cyclosporine conjugated compound of formula (I) which comprises esterifying a compound of the following formula (II) with a base to give a compound of the following formula (III) and then reacting the resulting compound (III) with polyethylene glycol derivatives of the following formulas (IV), (V) or (VI), in the presence of sodium iodide, potassium carbonate or crown ether, respectively:



(I)

Y-CH_2O-C(=O)-O-C(=O)-OCH_2-Y (II)

$CsA\text{-}O\text{-}C(=O)\text{-}OCH_2\text{-}Y$ (III)

$OCH_3\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2O\text{-}C(=O)\text{-}(CH_2)_m\text{-}C(=O)\text{-}XH$ (IV)

$OCH_3\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2O\text{-}C(=O)\text{-}XH$ (V)

$OCH_3\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2O\text{-}XH$ (VI)

in which

R represents a group of formula (a), (b) or (c):

(a) $-C(=O)-OCH_2XC(=O)-(CH_2)_mC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$

(b) $-C(=O)-OCH_2XC(=O)-OCH_2CH_2-(OCH_2CH_2)_n-OCH_3$, or

(c) $-C(=O)-OCH_2X-CH_2CH_2-(OCH_2CH_2)_n-OCH_3$,

X represents O, S, or NH,

m is an integer of from 1 to 6,

n is an integer of 10 to 460,

Y represents a leaving group.

7. A pharmaceutical composition which comprises as an active ingredient a conjugated compound of the formula (I) as defined in claim 1 togehter with a pharmaceutically acceptable carrier.

8. A method of treating transplantation rejection or graft vs. host disease in a mammal in need thereof, which comprises administering an effective amount of a conjugated compound of the formula (I) as defined in claim 1 to said mammal.

9. A method of treating a fungal infection in a mammal in need thereof, which comprises administering an effective amount of a conjugated compound of the fomula (I) as shown in claim 1 to said mammal.

10. A method of treating rheumatoid arthritis in a mammal in need thereof, which comprises administering an effective amount of a conjugated compound of the formula (I) as shown in claim 1 to said mammal.

11. A method of treating a retenosis in a mammal in need thereof, which comprises administering an effective amount of a conjugated compound of the formula (I) as defined in claim 1 to said mammal.

12. A method of treating a pulmonary inflammation in a mammal in need thereof, which comprises administering an effective amount of a conjugated compound of the formula (I) as defined in claim 1 to said mammal.

Fig.1

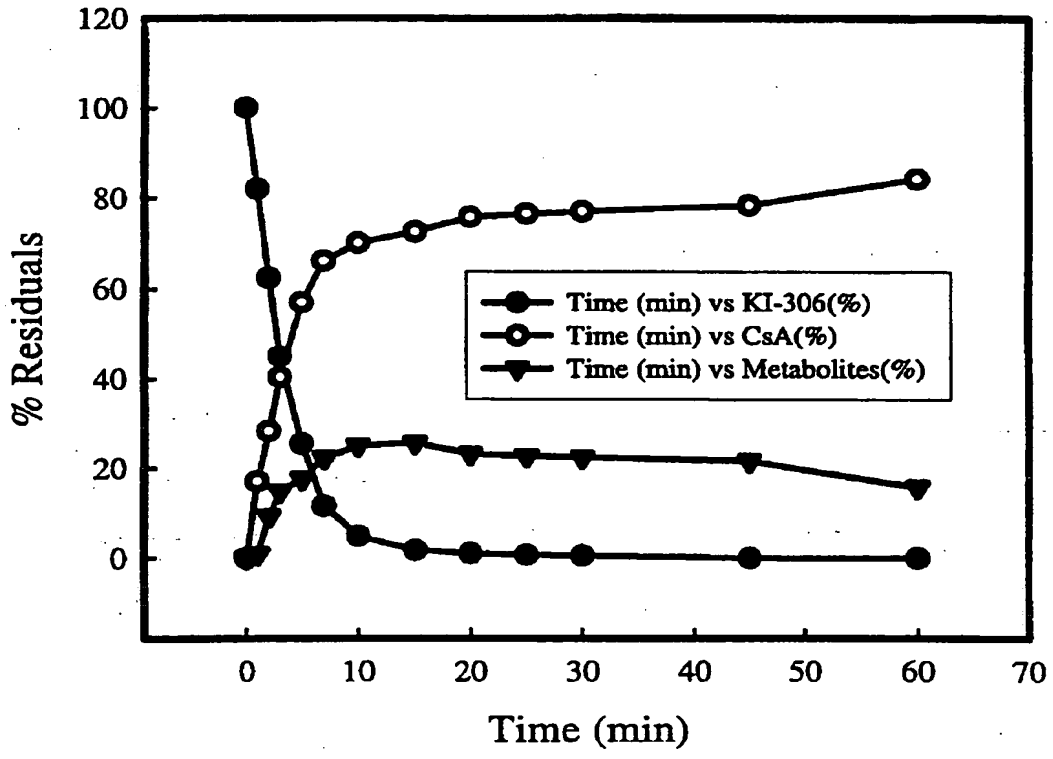

120
100
80
60
40
20
0
% Residuals
0
10
20
30
40
50
60
70
Time (min)
Time (min) vs KI-306(%)
Time (min) vs CsA(%)
Time (min) vs Metabolites(%)

Fig.2

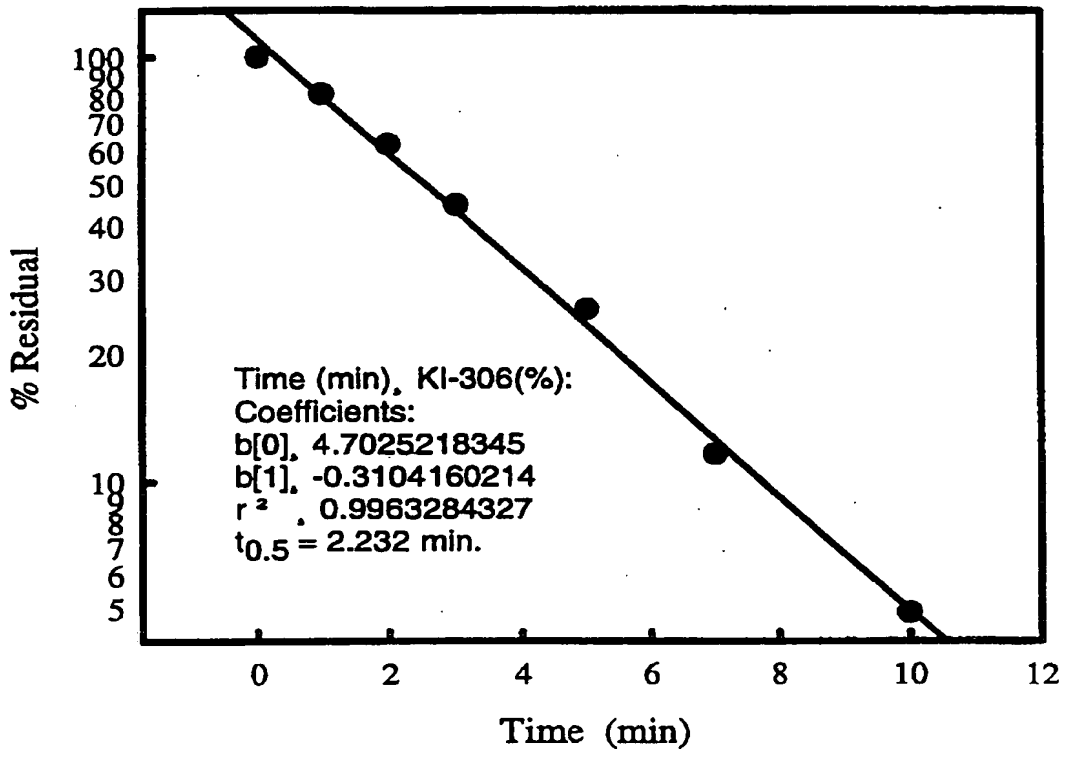

% Residual
100
90
80
70
60
50
40
30
20
10
8
7
6
5
0
2
4
6
8
10
12
Time (min)
Time (min), KI-306(%):
Coefficients:
b[0]= 4.7025218345
b[1]= -0.3104160214
r² = 0.9963284327
t0.5 = 2.232 min.

Fig.3

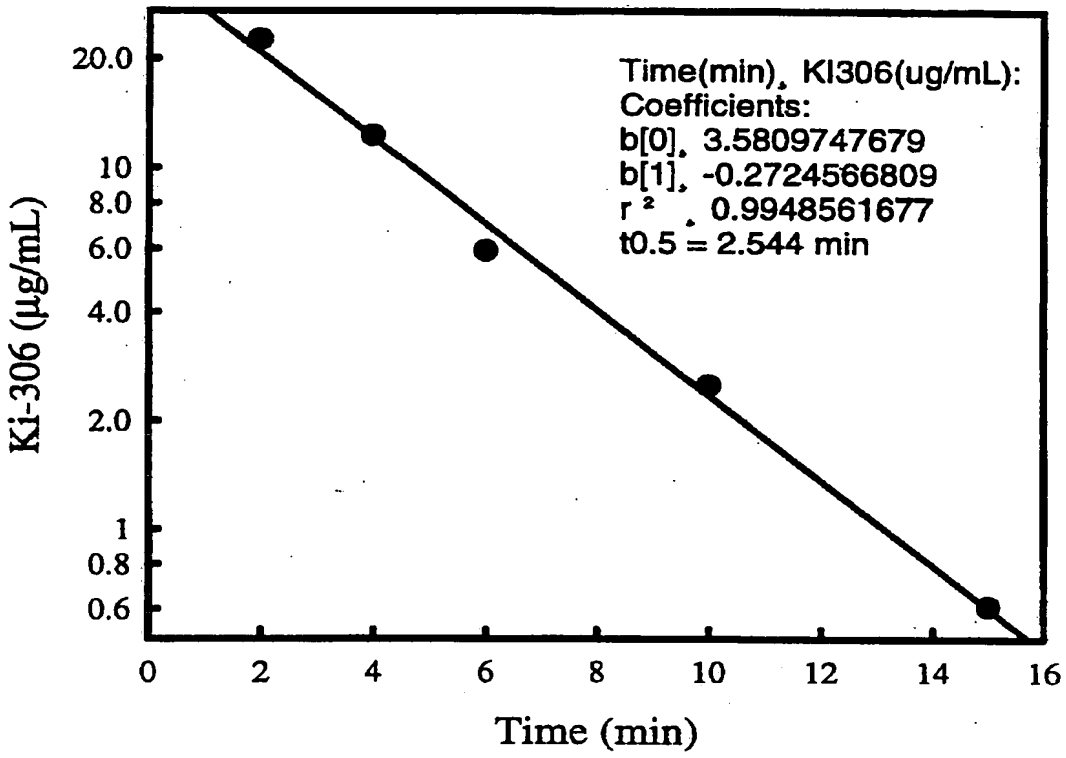
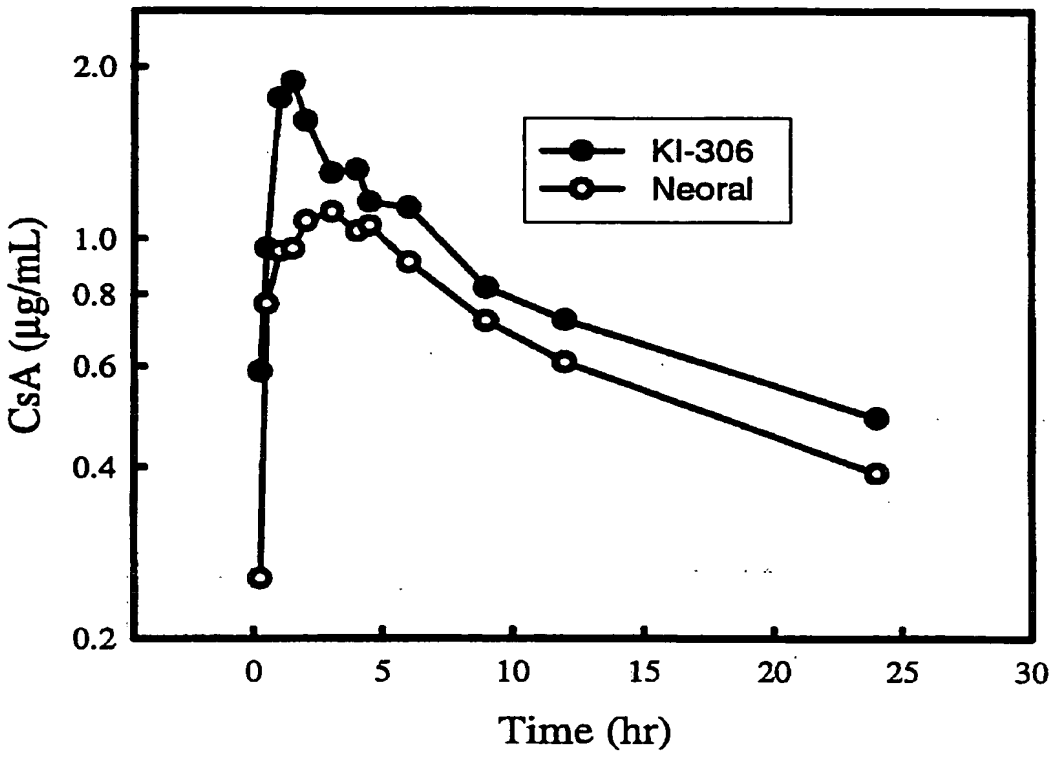

Time(min), KI306(ug/mL):
Coefficients:
b[0], 3.5809747679
b[1], -0.2724566809
r ², 0.9948561677
t0.5 = 2.544 min
Ki-306 (μg/mL)
20.0
10
8.0
6.0
4.0
2.0
1
0.8
0.6
0
2
4
6
8
10
12
14
16
Time (min)

Fig.4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00379

CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C 07 K 7/64; A 61 K 38/08; C 08 G 65/333

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C 07 K; A 61 K; C 08 G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL DATABASE, Derwent Publications Ltd., London (GB)
PAJ DATABASE, EPO PAJ Database
CA DATABASE, ST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98/07713 A1 (ENZON, INC.), 26 February 1998 (26.02.98) claims; example 41.	1-12
A	US 5614549 A (R.B. GREENWALD et al.) 25 March 1997 (25.03.97) claims; columns 5-9.	1-12
A	PAPRICA et al."Preparation of Novel Cyclosporin A Derivatives", Bioconjugate Chem., Vol.3, No.1, pages 32-36, totality.	1-12
A	WO 95/33490 A1 (ENZON, INC.) 14 December 1995 (14.12.95) pages 1-7; claims.	1-12

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed

„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 February 2000 (07.02.2000)	7 August 2000 (07.08.2000)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Weniger Telephone No. 1/53424/341

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00379

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remarks: A) Although the p resent claims 7-12 are directed to a method of treatment of the human or animal body by therapy (rule 39.1(iv)PCT), the search report has been established for these claims, as well, and based on the alleged effects of the composition.
B) Formula VI should be corrected, because in its present form its described a peroxy compund. Furthermore, in formulas IV-VI on page 10 of the application the methoxy terminated end of the PEG should be written in form CH3O-to avoid misunderstandings.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 99/00379

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO A1 9807713	26-02-1998	AU A1 40794/97	06-03-1998
		EP A1 923566	23-06-1999
		US A 5840900	24-11-1998
		US A 5965566	12-10-1999
		AU A1 80521/94	08-05-1995
		EP A1 727992	28-08-1996
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US A 5614549	25-03-1997	WO A1 9404193	03-03-1994
		AU A1 63612/94	26-09-1994
		WO A1 9420089	15-09-1994
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		EP A1 727992	28-08-1996
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		US A 5880131	09-03-1999
		US A 5965566	12-10-1999
		AU A1 49133/96	21-08-1996
		AU B2 705147	13-05-1999
		CA AA 2208841	08-08-1996
		EP A1 807115	19-11-1997
		EP A4 807115	07-10-1998
		JP T2 10513187	15-12-1998
		WO A1 9623794	08-08-1996
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